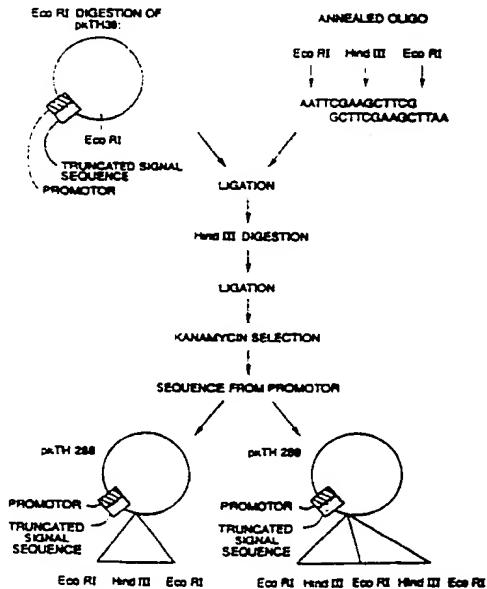




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<p>(21) International Application Number: PCT/FI91/00212</p> <p>(22) International Filing Date: 5 July 1991 (05.07.91)</p> <p>(30) Priority data: 903414 6 July 1990 (06.07.90) FI</p> <p>(71) Applicant (for all designated States except US): THE FINNISH NATIONAL PUBLIC HEALTH INSTITUTE [FI/FI]; Mannerheimintie 166, SF-00300 Helsinki (FI).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only) : SARVAS, Matti [FI/FI]; Pirttipolku 18 A, SF-00630 Helsinki (FI). BUTCHER, Sarah [GB/FI]; Servin-Maijan tie 10 D 47, SF-02150 Espoo (FI). NURMINEN-KALLIOKOSKI, Marjatta [FI/FI]; Vuolahdentie 5, SF-00950 Helsinki (FI). RUNEBERG-NYMAN, Kate [FI/FI]; Urheilukatu 36, SF-00250 Helsinki (FI). MUTTILAINEN, Susanna [FI/FI]; Melkonkatu 4 C 65, SF-00210 Helsinki (FI). WAHLSTRÖM, Eva [FI/FI]; Tehtaankatu 34 D, SF-00150 Helsinki (FI). IDÄNPÄÄN-HEIKKILÄ, Ilona [FI/FI]; Merimiehenkatu 35 A 9, SF-00150 Helsinki (FI). PUOHINIEMI, Ritvalleena [FI/FI]; Virtakuja 2c55, SF-01600 Vantaa (FI).</p>		<p>(74) Agent: OY KOLSTER AB; Iso Roobertinkatu 23, P.O. Box 148, SF-00121 Helsinki (FI).</p> <p>(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN + (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.</p> <p><b>Published</b> With international search report.</p>	
<p>(54) Title: PRODUCTION OF OUTER MEMBRANE (OM) PROTEINS IN GRAM-POSITIVE BACTERIA AND RECOVERY OF PROTECTIVE EPITOPE</p> <p>(57) Abstract</p> <p>The invention provides a method for producing cloned outer membrane (OM) protein from pathogenic gram-negative bacteria. The invention also provides a method for renaturing the cloned outer membrane protein thus produced so the cloned OM protein regains immunologically active epitopes which are capable of eliciting production of antibodies, in mammals and other animals, that are bactericidal and can provide protection against infection by the pathogenic gram-negative bacteria. According to the method, DNA encoding outer membrane protein from gram-negative bacteria, known to be pathogenic in humans and animals, is expressed in a gram-positive bacterial host. The recombinant or cloned OM protein thus produced is then renatured so as to regain biologically or immunologically active epitopes which are capable of eliciting production of antibodies in animals and humans; the antibodies are bactericidal and protect the animals and humans from infection by the pathogenic gram-negative bacteria from which the gene encoding the cloned OMP's was derived. The method of the invention is exemplified in part by the production of cloned and renatured class 1 outer membrane protein from <i>Neisseria meningitidis</i>, class 3 OM protein of <i>Neisseria meningitidis</i> and the OM protein OmpA of <i>Escherichia coli</i>.</p>			



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**+ DESIGNATIONS OF "SU"**

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PRODUCTION OF OUTER MEMBRANE (OM) PROTEINS IN  
GRAM-POSITIVE BACTERIA AND RECOVERY OF PROTECTIVE  
EPITOPES

5

Background of the Invention

The surface layer of gram-negative bacteria is composed of the outer membrane (OM). The bulk of OM is composed of so-called outer membrane proteins (OMP's). OMP's are proteins of unique structure and properties. In their native state the OMP's of gram-negative bacteria are 10 intimately bound to lipopolysaccharide (LPS) and other membrane components. The conformation of the OMP's seems to depend on their association with LPS's and other specific factors in the environment. What these other factors are, and how they affect conformation, are not well understood.

15 X-ray diffraction studies indicate that the epitopes on native proteins comprise about 15-25 amino acid residues, which are made up of two or three discontinuous surface loops. See for example, Laver *et al.*, *Cell* 61:553-556 (1990). The protective epitopes contained in the outer membrane proteins are exposed on the cell surface of the bacterium, where they are 20 capable of inducing antibodies that can protect an animal against infection by that strain of bacteria. Energetic calculations suggest that a smaller subset of 5-6 amino acid residues of an epitope contributes most of the binding energy to the antibodies, with the surrounding residues merely aiding in complementarity. The residues proposed to contribute most of the binding 25 energy are not arranged in a linear sequence but are scattered over the epitope surface.

In the OMP's, the epitope regions are loops connecting so-called beta-regions which protrude out of the membrane. These loops are hydrophilic and may be locally water soluble, even though this is not true of 30 the whole OMP.

In denatured proteins, the conformation of the native (protective) epitopes is usually lost, and antibodies formed against such denatured proteins are not protective against organisms from which such

proteins were derived. It is, however, sometimes possible to regain the proper conformation of the proteins (and their epitopes). Much experimental work has been done using heat, acid, alkali, urea and guanidine hydrochloride denatured proteins to elucidate the process of refolding. Small proteins 5 refold fast (e.g., 0.1 s) unless the *cis-trans* isomerization of proline residues or the formation of disulfide bonds is involved (Kim, P. and Baldwin, R. *Ann. Rev. Biochem.* 59:631-660 (1990)). The refolding of large proteins is naturally more complicated and very much more unpredictable. Proteins denatured with urea, guanidine hydrochloride or SDS easily loose the original *cis-trans* 10 configuration of their proline residues. The original configuration is not easily regained by chemical methods, but can sometimes be catalyzed by peptidyl-prolyl *cis-trans* isomerase enzymes (Fisher, G. and Bang, H. *BA* 828:39-42 (1985)). Restoration of the original disulfide bonds is also often 15 very problematic (Ewbank, J. and Creighton, T. *Nature* 350:518-520 (1991)). This can be true even in cases where the native proteins are water soluble. (See generally, Richard, F. *Scientific American* 264:34-41 (January, 1991), for more about the protein folding problem.)

Purified outer membrane proteins could be used in medicine as vaccines to prevent diseases caused by pathogenic gram-negative bacteria or 20 as reagents to diagnose such diseases by immunological methods. For example, *Neisseria meningitidis* bacteria (meningococci) cause a serious human infection, purulent meningitis (Peltola, H. *Rev. Infect. Dis.* J. 5:71-91 25 (1983)), and the need for a vaccine to prevent meningococcal infections has existed for a long time. In the early 1970's, capsular polysaccharide vaccines were shown to be efficacious against two meningococcal serogroups, A and C (World Health Organization Study Group. Technical Report Series No. 588. World Health Organization, Geneva, 1976). The capsule of the third major serogroup, B (hereinafter MenB) proved, however, to be structurally closely similar to the saccharide part of glycoproteins in some human tissues (Finne, 30 J., Leinonen, M., and Mäkelä, P.H. *Lancet* ii:355-357 (1983)). The resulting strong immunological cross-reactivity may explain why attempts to produce a capsular polysaccharide vaccine for MenB have failed.

Alternative candidates for a MenB vaccine have been sought. The outer membrane of *Neisseria meningitidis* bacteria, as the surface structure of the bacteria in immediate contact with the environment, is one such candidate. Protection afforded by monoclonal antibodies directed to different OM components has been studied in an infant rat model (Saukkonen, K. *Microbial Pathogenesis* 4:203-212 (1988)). Antibodies directed to OM proteins were found protective. OM complex vaccines, consisting of semi-purified OM components, have been prepared and tested in several field trials (Frasch, C.E. *Vaccine* 5:3-4 (1987); Frøholm, L.O., Berdal, B.P., Bovre, K., Gaustad, P., Halstensen, A.I., Harboe, A., Harthug, S., Holten, E., Høiby, E.A., Lystad, A., Omland, T., Rosenqvist, E., Viko, G., Frasch, C.E., and Zollinger, W.D. *Antonie van Leeuwenhoek* (52:239-241 (1986)).

Although there is a need for OMP's such as OMP's from *Neisseria meningitidis*, and other pathogens such as *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Yersinia sp.*, and *Brucella sp.*, their preparation and purification from gram-negative bacteria is difficult with conventional biochemical methods. A special problem is the tight association of the OMP's with lipopolysaccharide (Hitchcock, P.J., and Morrison, D.C. in E.T. Rietschel (ed.) Handbook of Endotoxin, vol. I. Chemistry of Endotoxin, Elsevier Science Publishing, Inc. New York, 1984.), which is toxic. The problem of removing toxic LPS has so far not been solved in a satisfactory manner, even using harsh methods.

Both the production and purification of outer membrane proteins would be simplified and more effective if the OMP's were produced using the methods of genetic engineering, in gram-positive bacteria, which are devoid of lipopolysaccharide.

When proteins are produced using the methods of genetic engineering, it is often a goal to produce the recombinant proteins in large amounts. Sometimes when large amounts of recombinant proteins are produced in bacteria, the proteins form insoluble aggregates. These insoluble aggregates are referred to as inclusion bodies. In inclusion bodies the

proteins are in an unnatural state devoid of their authentic configuration and epitopes. Before restoration of the biological activity (e.g., enzymatic or receptor activities, or protective epitopes) the recombinant proteins must be returned to their native conformations, i.e., need to be correctly folded.

5            Chaotropic agents and detergents can be used to solubilize some proteins and return them to their native conformations. (Chaotropic agents prevent the random coils among hydrophilic regions, which are characteristic of denatured lipophilic proteins in aqueous solutions, from occurring.) Unfortunately, the proteins produced in bacteria as inclusion  
10          bodies are very resistant to many chaotropic agents and detergents (see generally, *Methods of Enzymology*, 1990) and are soluble, if at all, only in high concentrations of urea or guanidine hydrochloride, or SDS. This is true even though the native proteins were water soluble.

15          In some cases the activity of an enzyme protein can be regained after removal of the chaotropic agent, e.g., active prourokinase is formed from inactive prourokinase inclusion bodies by solubilization with 6 M guanidine hydrochloride and 2-mercaptoethanol and subsequent refolding of the protein for 24 h at 15°C in a buffer containing 2 M urea (Orsini, G. et al., *Eur. J. Biochem.* 195:691-697 (1991)). Some recombinant proteins are  
20          soluble in sarkosyl (Puohiniemi, R., M. Karvonen, J. Vuopio-Varkila, A. Muotiala, I.M. Helander and M. Sarvas. *Int. Imm.* 58:1691-1696 (1990); Frankel, S. et al., *Proc. Natl. Acad. Sci* 88:1192-1196 (1991)) and may regain their biological activity after removal of the detergent. Alkali may also induce native conformation of inclusion body proteins. Prochymotrypsin,  
25          solubilized in alkali-urea, folds in a proper native conformation and is subsequently able to autoprocess at an acidic pH (Marston, F. et al., *FEMS Microbiol. Letters* 77:243-250 (1984)).

Less is known about the restoration of epitopes on denatured antigens than is known about the recovery of enzymatic activity in proteins  
30          that function as enzymes. What little knowledge there is has come from studies involving denatured native proteins. In one study outer membrane proteins, OmpA and OmpF of *E. coli*, were first purified by preparative

SDS-gel electrophoresis (Dornmair, K. et al., *J. Biol. Chem.* 265:18907-18911 (1990)); in another these same OMP's were extracted with octyl-POE extraction (Eisele and Rosenbusch, 1990). Lipopolysaccharide (LPS) was not required to restore activity in either case. This is not in accordance with a 5 study with *E. coli* spheroplasts (Sen, K. and Nikaido, H. *J. Bacteriol.* 173:926-928 (1991)), which showed that trimerization of OmpF protein takes place only in the presence of LPS.

While the knowledge of the renaturing of *Escherichia coli* OmpA and OmpF is a useful addition to the store of overall knowledge of 10 renaturation of native proteins, it does not teach or predict what is required for renaturation of recombinantly produced OMP's that never were in tight association with native LPS's or other membrane and/or environmental components.

As indicated above, the outer membrane proteins of gram-negative bacteria are intimately bound to lipopolysaccharide (LPS) and perhaps other membrane components; their proper conformation is dependent on this association with such components of the membrane environment. Thus if LPS and possibly other membrane components are not present in preparations of isolated OMP's, these stabilizing components must 15 be replaced by other components that mimic their function, so as to ensure the stability of the OMP in its native beta-barrel conformation. (The beta structure is the well-ordered part of the OMP that is complexed with LPS.) 20

Thus renaturation of OMP's is more complicated than merely solubilizing a recombinantly produced OMP and dialyzing out the chaotropic agent. To restore immunogenic function, i.e., to re-stabilize the membrane-bound regions that normally exist in a beta-configuration so as to expose the epitopic loops, it is necessary to replace the detergent or other agent with an 25 the environment that mimics the natural one. While it may not be necessary to recover 100% of the native OMP conformation, this replacement must permit (a) proper refolding of the epitopic loops (which are a relatively small 30 part of the total peptide), and (b) their accessibility to the immune system, i.e., the epitopic loops must protrude into the primarily aqueous environment

after injection into an animal or human and subsequent dilution of the preparation.

It is an object of the present invention to provide a method for producing pure cloned outer membrane proteins, and to provide a method 5 for their renaturation so as to regain biologically or immunologically active epitopes which are capable of eliciting the production of antibodies in animals that are (a) bactericidal and (b) protect the animals against infection by the original infectious agent.

It is a further object of the present invention to use these pure 10 cloned and renatured OMP's as diagnostic antigens for the identification of infections caused by gram-negative bacteria.

It is a further object of the present invention to use these pure cloned and renatured OMP's in vaccines to protect animals and humans 15 against infection by that strain of bacteria from which the gene encoding the cloned OMP's was derived.

#### Definitions

In the present specification and claims, reference is made to phrases and terms of art which are expressly defined for use herein as 20 follows:

By "regulation and expression sequence" it is meant to include within the scope of the instant invention the DNA sequence of a gene preceding the DNA sequence encoding a polypeptide; the DNA sequence is needed for the transcription and translation of the DNA sequence encoding 25 that polypeptide. Such sequence typically includes the promoter and ribosomal binding site and possibly binding sites for regulatory proteins. The "regulation and expression sequence" may be any biologically active fragment thereof. "The regulation and expression sequence" may include also a DNA sequence encoding an N-terminal fragment of the polypeptide, if that 30 fragment of the polypeptide is not a functional signal sequence for export.

By "outer membrane protein" and by "mature outer membrane protein" it is meant an outer membrane protein, which is devoid of a signal peptide for export, or devoid of a functional signal peptide.

5 By "vector" it is meant any autonomous element capable of replicating in a host independently of the host's chromosome, into which additional sequences of DNA may be incorporated. Such vectors include, but are not limited to, bacterial plasmids and phages.

10 By "operationally linked" it is meant that the regulation and expression sequence, including the promoter, controls the initiation of expression of the polypeptide encoded by the structural gene; there may be a DNA sequence derived from the same gene as the promoter or any other DNA sequence between the promoter and the initiation of the polypeptide to enhance the expression of the polypeptide. This DNA sequence may encode a peptide that remains fused to the polypeptide, but the said peptide must 15 not be a functional signal for export.

As used herein, "recombinantly produced", when referring to the production of OMP, means that the OMP's of one species of bacteria, or DNA encoding for such protein, are expressed in the cells of a bacterium from another species. Recombinantly produced OMP will have been 20 produced using the techniques of gene expression and genetic engineering. Recombinantly produced OMP's are cloned OMP's, and are to be distinguished from OMP's that are extracted from the natural outer membranes of bacteria.

25 **Summary of the Invention**

The invention provides a method for producing cloned outer membrane (OM) protein from pathogenic gram-negative bacteria. The invention also provides a method for renaturing the cloned outer membrane protein thus produced so the cloned OM protein regains immunologically active epitopes which are capable of eliciting production of antibodies, in mammals and other animals, that are bactericidal and can provide protection 30 against infection by the pathogenic gram-negative bacteria. According to the

method, DNA encoding outer membrane protein from gram-negative bacteria, known to be pathogenic in humans and animals, is expressed in a gram-positive bacterial host. The recombinant or cloned OM protein thus produced is then renatured so as to regain biologically or immunologically active epitopes which are capable of eliciting production of antibodies in animals and humans; the antibodies are bactericidal and protect the animals and humans from infection by the pathogenic gram-negative bacteria from which the gene encoding the cloned OMP's was derived. According to the invention, a *Bacillus* or other suitable gram-positive bacterial host containing a recombinant DNA molecule comprising the regulation and expression sequence of a gene expressed well in the host is operationally linked to a DNA sequence encoding an outer membrane protein from a gram-negative bacteria known to be pathogenic to animals and humans. The regulation and expression signal is typically an effective promoter and ribosomal binding site.

In preferred form, the DNA sequence encoding the outer membrane protein is devoid of functional signal sequence, as is the regulation and expression sequence. The presence of signal sequences decreases the amount of recombinant outer membrane protein expressed in the gram-positive host.

According to the invention, the recombinant DNA molecule may be introduced into the *Bacillus* or other suitable gram-positive host by transforming the host with a vector that is capable of replicating in several copies in the host strain. Alternatively, the recombinant DNA molecule may be integrated into the chromosome of the *Bacillus* or other host strain. Using the method, more than one hundred milligrams of product per liter of culture are obtained when ordinary laboratory media are used. The amount can be higher in high density cultures. The product may be aggregated intracellularly or found in the form of inclusion bodies.

According to the teaching of the invention, the conditions for refolding the OM proteins are such that allow the OM proteins to take the same or partially the same conformation as they have in their natural environment, in the outer membrane. While such conditions can be achieved

by using the same amphiphilic compounds present in OM, e.g., LPS, use of LPS is not preferred since LPS is toxic. Non-toxic derivatives of LPS can be used. Other non-toxic lipids or their derivatives or analogs, may also be used, alone or in conjunction with detergents, variations in pH and/or temperature, 5 the addition of chemicals, e.g., sugars and amino acids, as well as other conditions which may favor refolding of protective epitopes. Any of these methods and agents, or combinations of agents, may be used as long as they permit (a) proper refolding of the epitopic loops and (b) their accessibility to the immune system, i.e., the epitopic loops must protrude into the primarily 10 aqueous serum of an animal or human after injection and subsequent dilution.

The method of the invention is exemplified by the production of cloned and renatured class 1 outer membrane protein from *Neisseria meningitidis*, class 3 OM protein of *Neisseria meningitidis* and the OM protein 15 OmpA of *Escherichia coli*, all in *Bacillus subtilis*. However, as those skilled in the art will appreciate, as a result of the teaching that it is possible to renature recombinant OM proteins, the method can be used to produce other outer membrane proteins, including, but not limited to, other OM proteins of *Neisseria meningitidis*, and the OM proteins of *Neisseria gonorrhoeae*, 20 *Haemophilus influenzae*, *Yersinia sp.* and *Brucella sp.*

One objective of the invention is to use the method for the production of safe and effective vaccines. Another objective is to provide diagnostic antigens for the identification of infections caused by gram-negative bacteria.

25

#### Brief Description of Drawings and Nucleotide Sequences

Figure 1. Construction of pKTH288 and 289.

Figure 2. Construction of pKTH290.

30 Figure 3. The protein pattern of whole cells of *Bacillus subtilis* strains. Lane a, low molecular weight standards, lanes b, c, d and f control strains, lane e, IH6627.

Figure 4. The protein pattern of inclusion bodies (=2,000xg pellets) derived from 0.5 mg wet weight of bacteria. Lane a, low molecular weight standards, lanes b, c, d and f, control strains, lane e, IH6627.

5       Figure 5. The sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE, Coomassie Blue staining) of 12 transformant colonies tested (see Example 3). Lane 1, the molecular weight standard; Lane 2, Sarkosyl solubilized Bac-OmpA produced from pKTH217 (Puohiniemi, R., M. Karvonen, J. Vuopio-Varkila, A. Muotiala, I.M. Helander and M. Sarvas. *Inf. Immun.* 58:1691-1696 (1990); Lane 3, Mock 10 preparation made similarly to the OmpA preparation in lane 2 from IH6418, a strain containing the secretion vector without any insert. Lanes 4-15, samples of separate transformant colonies. The figures to the left show the position and size (kDa) of molecular weight markers. Symbol on the right indicate position of OmpA.

15       Figure 6. SDS-PAGE and Coomassie Blue staining of different steps of particulate centrifugation of 500 ml of IH6649 (wet weight of cells 5-7 g). Lane 1, the molecular weight standards, 5  $\mu$ l (suspended in 500  $\mu$ l of sample buffer) was applied; Lane 5, the supernatant after 2,000xg centrifugation, 2  $\mu$ l of 20 ml was applied; Lane 6, the pellet after 2,000xg 20 centrifugation (wet weight 1.88 g), the pellet was resuspended in 10 ml of 50 mM TrisHCl (pH 8) and 0.3  $\mu$ l was applied; Lane 13, the 2,000xg pellet after washing (wet weight 0.38 g), the final pellet was resuspended in 4 ml of the above buffer and 3  $\mu$ l of a 1 to 10 dilution was applied; Lane 14, the pellet after 5,000xg centrifugation (wet weight 0.45 g), the pellet was resuspended in 25 5 ml of the above buffer and 3  $\mu$ l of a 1 to 10 dilution was applied; Lane 11, the supernatant after 5,000xg centrifugation, 2  $\mu$ l of the 20 ml supernatant was applied. The figures to the left show the position and size (kDa) of molecular weight markers. Symbols on the right indicate position of OmpA.

Sequence ID Numbers 1 and 2 (Seq. ID 1 and Seq. ID 2).

30       Oligonucleotides used to amplify the DNA coding for the class 1 proteins in a PCR reaction. The oligonucleotide of Seq. ID 1 consists of 4 nucleotides (AACG), a *Hind*III site, and nucleotides 125-155 of Barlow, *et al.*, *Mol.*

*Microbiol.* 3:131-139 (1989) coding for the first 10 amino acids of the mature protein. The oligonucleotide of Seq. ID 2 consists of 4 nucleotides (AACC), the reverse sequence of the following: a *Hind*III site (including part of the stop codon), stop codon and nucleotides 1246-1217 of Barlow, *et al.*, *Mol.*

- 5 *Microbiol.* 3:131-139 (1989). The sequences are included in the specification, just preceding the claims.

Sequence ID Number 3 (Seq. ID 3). DNA sequence of the pKTH250 insert; P1.7,16. Nucleotide 1 of the sequence shown, corresponds to nucleotide 125 of the sequence from Barlow *et al.*, *Mol. Microbiol.* 3:131-  
10 139 (1989).

#### Detailed Description of the Invention

##### Production of OM proteins

As those skilled in the art will appreciate, the methods described in the following paragraphs for *Bacillus* can be applied with appropriate modifications, if needed, and without undue experimentation, for other gram-positive bacteria.

##### 20 The Production of OM Proteins by Transforming a *Bacillus* or Other Gram-Positive Host

A wide variety of suitable expression vectors may be used in the present invention, and are known to those of skill in the art of recombinant genetics. A preferred vector is disclosed in PCT WO 90FI41, filed 2 Feb 1990 and published 23 Aug 1990 as WO 9009448: New Recombinant DNA  
25 Molecules for Producing Proteins and Peptides in *Bacillus* Strains.

The transfer vector may be any plasmid or phage capable of replicating in several copies in a *Bacillus* strain or other gram-positive bacterium. A multitude of such vectors are available, the most representative of them being the plasmids isolated from *Staphylococcus*, *Bacillus* or  
30 *Streptococcus* or their derivatives.

The regulation and expression sequence is in most cases first ligated to the transfer vector to be used and is thereafter modified for

example by the aid of DNA-linkers so that the genes to be expressed may be joined downstream from the regulation and expression sequence of the vector.

5 A number of methods to clone OM protein genes can be used in the present invention. Those methods are known to those of skill in the art of recombinant genetics.

Before transformation of the host, a DNA sequence encoding the OM protein (or epitopically functional portion(s) thereof) is ligated to a suitable vector.

10 The DNA sequences need not be identical to the DNA sequences encoding a particular OM protein as found in a particular natural gene. They may be derived from the sequences of natural genes of OM proteins, but modified in ways that may alter the properties of the resulting protein. Suitable sequences may also be made synthetically or semi-synthetically.

15 The selected host may be transformed and cultivated by conventional methods. The choice of suitable transformation systems and cultivation conditions depends on the selected host.

20 Purification of OM Proteins Produced Intracellularly  
in *Bacillus* Host or in Other Gram-Positive Bacteria

The OM proteins produced with the method of the invention often form intracellular inclusion bodies. The main advantage of producing inclusion bodies (or intracellular aggregates of overproduced protein) is that they are easy to purify (Marsion and Hartley, *Methods of Enzymology* 182:264-276 (1990)). The bacterial cells are disrupted by sonication, passage through French pressure cells, lysozyme-treated or by other suitable means. From this suspension the inclusion bodies can be pelleted with low speed centrifugation and usually further washed with a mild detergent. Usually (however, depending on the protein) the inclusion bodies are soluble only in chaotropic agents like urea or guanidine hydrochloride or strong detergents

like SDS. In the solubilized form, the proteins can be further purified with conventional purification methods.

If the OM protein produced in *Bacillus* is not present in inclusion bodies, modifications of the methods above may be applied. The 5 purification may also involve solubilization and differential extraction with various types of detergents, and chromatography and electrophoresis in the presence and absence of detergents.

There are several different ways known to those skilled in the art, in which a composition of the recombinant polypeptides produced by the 10 method of the invention may be prepared. The purified OM proteins or their fragments may be used alone to prepare a pharmaceutically-acceptable dosage form and they may be mixed together in any combination. The recovery of the native epitopes may involve addition of solubilization and/or denaturing agents such as urea, guanidine hydrochloride and SDS, which may 15 be later removed. It may also involve addition of compounds like phospholipids and/or sarkosyl or their derivatives and analogs. The preparation may be in a form of liposomes or in another form. Immunoadjuvants such as aluminium hydroxide and pharmacologically-acceptable preservatives such as thiomersal may be added 20 to the composition. These methods are described, for example, in Remington Pharmaceutical Science, 16th Ed., Mac. Eds. (1980).

Without further elaboration, it is believed that one of ordinary skill in the art can, using the preceding description, and the following Examples, utilize the present invention to the fullest extent. The material 25 disclosed in the examples is for illustrative purposes and therefore should not be construed as being limiting in any way of the appended claims.

## Examples

## Example 1

Cloning of P1.7,16 (class 1) OM Protein of *N. meningitidis* in IH6627

## Cloning of the DNA Sequence Coding for the Class 1 Protein

5       The DNA fragment coding for the mature protein was acquired as follows: using the published nucleotide sequence of meningococcal class 1, P1.7,16 protein (Barlow *et al.*, *Mol Microbiol* 3:131-139 (1989)) two oligonucleotides (primer 1 and primer 2; primer 1 is shown herein as Seq. ID 1; primer 2 is Seq. ID 2) were synthesized and used to amplify the DNA  
10      coding for the mature class 1 protein in a polymerase chain reaction (PCR) with chromosomal meningococcal DNA. The meningococcal DNA was isolated from strain IH5341 (P1.7,16) grown on protease-peptone plates containing 1.5% agarose in lieu of agar. After treatment with a zwitterionic detergent in citrate buffer (Domenico *et al.*, *J Microbial Methods* 9:211-19  
15      (1989)) to remove e.g., capsule, the DNA was isolated. The PCR reaction was performed using a GeneAmp™ kit using the methods described by the manufacturer (Perkin Elmer Cetus). The amplified DNA fragments were of two sizes when separated by agarose gel electrophoresis. The bigger fragment seemed to be the expected size for class 1, i.e., about 1100 bp  
20      whereas the other fragment was smaller, about 900 bp. The amplified DNA mixture was purified by phenol extraction, ethanol precipitated, resuspended in TE (10 mM Tris-HCl pH8, 1 mM EDTA) and digested with the restriction enzyme *Hind*III. Conventional methods of DNA technology and  
microbiology used here and in the following examples are described, e.g., in  
25      Sambrook, J., E. F. Fritsch, and T. Maniatis. (1989) Molecular Cloning. A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, N.Y.

30      The plasmid vector pUC18 was digested with the restriction enzyme *Hind*III. The linearized vector DNA was ligated with the *Hind*III-digested amplified DNA. The ligation mixture was used to transform competent *Escherichia coli* K12 TG1 cells which were grown, after transformation, on Luria plates containing 100 µg/ml ampicillin, 40 µg/ml Xgal and 0.5 Mm IPTG. About 10% of the colonies grown overnight were

blue, thus representing the background caused by the vector. In the case of the amplified P1.7,16 DNA, 90 white colonies were tested to check the size of the putative insert. Of these, 11 contained a plasmid with an insert of the expected size. One of these strains, EH1563, containing plasmid pKTH250, 5 was further characterized. It was shown to give the expected sized fragments after treatment with the restriction enzymes *Hind*III, *Eco*R1 or *Kpn*I. The insert was sequenced with the Sanger dideoxy sequencing method after subcloning into M13. The sequence of the pKTH250 insert is shown as Seq. ID 3. As expected it shows very few changes compared to the published 10 P1.7,16 sequence (Barlow *et al.*, *Mol. Microbiol.* 3:131-139 (1989)).

Construction of pKTH290 for Intracellular Production  
of Class 1 OM Protein in *Bacillus subtilis*

Construction of the plasmid pKTH290 is shown schematically in Fig. 3. Plasmid pKTH250 (pUC18 containing DNA coding for the cloned class 1 protein P1.7,16) was digested with *Hind*III to release the cloned class 1 gene. Also plasmid pKTH289 was digested with *Hind*III to release the extra adapter copy and to linearize the vector (see Fig. 1). The two *Hind*III digested plasmids were ligated and the ligation mixture was used to transform 15 IH6140. Cells that had received at least pKTH289 were selected on the basis of kanamycin resistance. The size of plasmids present in the colonies which grew on Luria plates containing kanamycin was checked by cell lysis and running samples in an agarose gel by standard methods. Colonies which 20 contained plasmids of the expected size were analyzed for class 1 protein expression by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE followed by immunoblotting (Western blot). 25 One strain IH6627 containing plasmid pKTH290 which expressed the class 1 protein was further analyzed. The cloning site was checked by Sanger dideoxy sequencing of pKTH290 to ensure that only one copy of the adapter 30 was present.

Screening for the Expression of Class 1 Proteins  
Produced Intracellularly in *Bacillus subtilis*

The expression of class 1 P1.7,16 protein in *Bacillus* transformants was screened using SDS-PAGE in the following way: a loopful of bacteria grown on Luria agar plates was suspended in Laemmli sample buffer and after heating at 100°C, a sample was applied to SDS-PAGE. The class 1 protein was visualized with Coomassie Blue staining (Fig. 3) and with immunostaining of the SDS-PAGE after blotting the proteins onto millipore filter (Western blot). Antisera KH 1110 prepared by immunization of a rabbit with an extract of MenB:15:P1.7,16 bacteria, was used in immunostaining. The result was confirmed with a P1.7,16 specific monoclonal antiserum, obtained from a commercial kit for serotyping meningococci (RIVN, Box 457, 3720 AL Bilthoven, The Netherlands).

One transformant expressing P1.7,16 protein (strain IH6627) was chosen for further studies.

Similar constructions were also made with DNA coding several other class 1. protein subtypes (P1.15, P1.2, Pl.I, Pl.9) and meningococcal class 3 protein serotypes 9 and 15.

20

Example 2

Production of BacP1.7,16 Protein, the P1.7,16 (class 1) OM Protein  
of *N. meningitidis* in IH6627

25

Preparation of Inclusion Bodies (IB) Containing BacP1.7,16 Protein

The bacteria were grown either in liquid or on solid Luria medium containing 10 - 30 µg kanamycin per liter. When growing one liter of Luria broth about 10 g bacterial cells (wet weight) were obtained.

30

The bacteria were disrupted with lysozyme in the following way: one gram of bacteria grown on Luria agar plates containing kanamycin was suspended in 5 ml of 20 sucrose in buffer (25 mM TrisHCl, pH 8.0; 15 mM MgCl<sub>2</sub> containing 1 mg lysozyme/ml). After an incubation of 30 min at 37°C the protoplasts were collected by centrifugation (10,000xg) and lysed by

suspending them in 5 ml of 50 mM TrisHCl, pH 8.0. DNase (1 mg/ml) was added and after five minutes inclusion bodies were collected by centrifugation 10,000xg for 10 min. They were further suspended (washed) in 5 ml of 2 % NP-40 in 50 mM TrisHCl, pH 8.0, and collected by centrifugation 10,000xg for 10 min. A sample of the product was electrophoresed in SDS-PAGE (Laemmli). Fig. 4 shows the protein pattern of this SDS-PAGE stained with Coomassie Blue. BacP1.7,16 protein isolated as inclusion bodies was called BacP1.7,16-IB.

The inclusion body fraction derived from 10 g of bacteria (about 500 mg of protein) contained 300 mg protein, as measured by the Lowry method (Lowry, O.H. *et al.*, *J. Biol. Chem.* 193:265-275 (1951)). The amount of BacP1.7,16 protein in this fraction was roughly estimated by visual inspection of the SDS-PAGE (Fig. 6) to be at least 120 mg. That means that more than 1/3 of the protein present in the inclusion bodies from IH6627 is BacP1.7,16 protein. It can be calculated that BacP1.7,16 protein comprised at least 25% of the total cellular protein of IH6627. It can also be calculated that there was more than one hundred mg of BacP1.7,16 protein per liter of culture.

The size of the BacP1.7,16 protein is roughly the same as that of the authentic protein of *N. meningitidis* in SDS-Page.

### Example 3

#### Intracellular Production of Outer Membrane Protein

##### OmpA of *Escherichia coli* in *Bacillus subtilis*

25

##### Construction of the Expression Vector pKTH3125

The plasmid pKTH 217 (described in Puohiniemi, R., M. Karvonen, J. Vuopio-Varkila, A. Muotiala, I.M. Helander and M. Sarvas. *Inf. Imm.* 58:1691-1696 (1990)) contains a 2.5 Kb *Hind*III-BamHI fragment which encodes, starting at the *Hind*III terminus, the 8 to 325 amino acid residues of the OmpA protein of *E. coli*. The fragment is flanked at the *Hind*III terminus by a unique *Cla*I-*Hind*III fragment. To construct the plasmid

pKTH3125, this *Cla*I-*Hind*III fragment was replaced by the *Cla*I-*Hind*III fragment of the plasmid pKTH288 shown in Figure 1. The latter fragment contains the promoter and truncated, nonfunctional signal sequence of  $\alpha$ -amylase. In pKTH3125 they were fused to the DNA fragment encoding the OmpA protein. The plasmids pKTH217 and 288 were digested with endonucleases *Cla*I and *Hind*III, phenol extracted, ethanol precipitated and resuspended in water. Then 1.2  $\mu$ g of digested pKTH217 was mixed with 2.5  $\mu$ g of digested pKTH288, treated with polynucleotide kinase and then ligated. All DNA manipulations were performed as described in Maniatis *et al.*, (Maniatis, T., E. F. Fritsch, and J. Sambrook, Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, N. Y. 1982.) Competent cells of *B. subtilis* strain IH6140 were then transformed with the ligation mixture as described in Maniatis *et al.*, and plated on Luria-plates containing 10  $\mu$ g of kanamycin. The expression of OmpA was tested as follows from 24 of more than  $10^4$  transformant colonies obtained. About half of the colony was mixed with 10  $\mu$ l of SDS-PAGE sample buffer, heated to 100°C for 5 minutes and the sample was electrophoresed in SDS-PAGE (Laemmli, U. K. *Nature*, (London) 227:680-685 (1970)). As judged by the presence of a major band of the size of the mature OmpA protein (Fig. 4, lanes 4, 6-9, 13, 14), several colonies contained OmpA protein. This band also reacted with OmpA serum in immunoblotting. One of them was designated IH6649, and the plasmid in this strain pKTH3125.

#### Analysis and Purification of OmpA Made in IH6649

IH6649 was grown overnight at 37°C with shaking (250 rpm) in liquid culture in twofold-concentrated L-broth containing 10 mg of NaCl per liter, 10  $\mu$ g of kanamycin per ml, and 30  $\mu$ l of potato extract per ml (Kallio, P., M. Simonen, I. Palva, and M. Sarvas, *J. Gen. Microbiol.* 132:677-678 (1986)). Cells from 500 ml of culture were collected by centrifugation (wet weight 5.7 g), protoplasted with lysozyme and disrupted with osmotic shock in the presence of DNase and RNase (about 5  $\mu$ g/ml) (Schnaitman, C., Manual of Methods for General Bacteriology ASM, Washington DC (1981)). The

breakage of cells was monitored by phase contrast microscopy. The particulate material was then pelleted by centrifugation at 16,000xg, for 10 min. OmpA was not a major band in the supernatant, as analyzed with SDS-PAGE (Fig. 5, lane 4). The pellet (1.9g wet weight) was resuspended in 5 50mM TrisHCl, pH 8, and centrifuged at 2,000xg, for 5 min. The pellet (0.9 g wet weight) was resuspended in 10 ml of washing buffer containing 5 mM EDTA, 150 mM NaCl, 1% NP-40, 50 mM TrisHCl, pH 8. The SDS-PAGE of the suspension showed that it contained OmpA as a major band (Fig. 5, lane 6). The suspension was then centrifuged at 5,000xg, for 10 min. The 10 pellet was washed with PBS and pelleted again (5,000xg, 10 min). The resulting pellet, 0.38 g (wet weight), was resuspended into 4 ml 50 mM TrisHCl, pH 8 (Fig. 5, lane 13). The supernatant, after the above 2,000xg centrifugation (Fig. 5, lane 5) also contained OmpA as a major band and that is why it was further centrifuged at 5,000xg, for 10 min. This resultant: 15 supernatant contained only traces of the original OmpA (Fig. 5, lane 11) and the PBS-washed pellet (0.45g wet weight) was resuspended in 5 ml of 50 mM TrisHCl, pH 8 (Fig. 5, lane 14), also contained OmpA but a lesser amount (compare lanes 13 and 14 in Fig. 5 in which the same amount of sample was applied). As a conclusion, surprisingly, when OmpA is expressed in *B.* 20 *subtilis* IH6649 it forms aggregates or inclusion bodies that can be collected by centrifuging at 2,000-5,000xg.

The amount of OmpA in the 2,000xg pellet after washing and the 2,000xg supernatant after pelleting at 5,000xg and washing was estimated visually by comparing the intensity of the OmpA bands in SDS-PAGE (Fig. 5, 25 lanes 13 and 14) with the intensity of the molecular weight standard bands (when 5  $\mu$ l of the standard is applied the 67 kDa and 30 kDa band contain 0.83  $\mu$ g of protein, the 43 kDa band contains 1.47  $\mu$ g protein according to the manufacturer, Pharmacia). Into both lanes were applied 3  $\mu$ l of 1:10 diluted sample. The band in lane 13 was estimated to contain 1.5  $\mu$ g of OmpA 30 which makes the total amount 20 mg in the 2,000xg pellet. The band in lane 14 was estimated to contain 0.8  $\mu$ g of OmpA which makes the total amount

13 mg of OmpA in the 5,000xg pellet. The total yield of purified OmpA was thus about 60 mg/l of culture.

5                   Refolding of OM Proteins Produced in *Bacillus subtilis* and Recovery  
of Protective Epitopes from BacP1.7,16 Protein Produced in IH 6627

The presence of protective epitopes in refolded BacP1.7,16 protein was analyzed by immunizing mice and analyzing the immune sera in enzyme immunoassay (EIA), and in bactericidal and protection assays. In the case of OmpA the refolding of native epitope was analyzed by a  
10                 bacteriophage inhibition assay (Example 8).

Immunization of Mice

The recovery of protective epitopes was tested by immunizing groups of ten mice with 20 µg of BacP1.7,16 protein treated in various ways, 15 given in two injections. The immunizing injection was either subcutaneous or intraperitoneal (i.p.) with 0.1 ml of antigen diluted in PBS. The interval between the two doses was six weeks. The first injection contained an adjuvant as indicated in Tables 1-4. Ten days after the second injection the mice were bled, and the pooled sera analyzed.

Analysis of the Immune Sera

Enzyme Immunoassay (EIA)

Anti-meningococcal antibodies were measured by EIA (Jalonnen 25 *et al., J. Infect.* 19:127-134 (1989)) using P1.7,16 meningococcal OM preparation or BacP1.7,16 protein as the antigens. The optimal dose for coating was in both cases 5 µg protein/ml.

### Bactericidal Assay

(Goldschneider *et al.*, *J. Exp. Med.* 129:1307-1323 (1969)) *N. meningitidis* group B:15:P1.7,16 strain H44/76 (from E. Holten, Norway) was used in the bactericidal assay. Meningococci of other subtypes (strains 5 MenB:2b:P1.2:L2 and MenB:15:P1.15:L1,8 from J. T. Poolman, The Netherlands) were used to assess the specificity of the bactericidal reaction. Fresh quinea pig serum was used as complement source. The highest serum dilution that gave 50% killing was taken as the end point titer. It is known that the bactericidal activity of serum correlates with protection. Thus all 10 sera, which were positive in this assay, were also tested in protection assays.

### Assay for Protection Against MenB-Infection

The ability of the sera to protect infant rats from bacteremia and meningitis was tested in the experimental infection of 5 day old outbred 15 Wistar rat pups (Saukkonen, *Microb. Pathog.* 4:203-212 (1988)). A mouse serum (HH209) obtained by immunization with a P1.7,16 meningococcal OM preparation was used as a positive control.

The pups were randomized in groups of 6 pups each and injected i.p. with 100  $\mu$ l of the immune sera in several dilutions (1:10, 1:100, 20 1:1000). One hour later a bacterial challenge  $10^6$  bacteria/ml) was injected i.p. in a volume of 100  $\mu$ l. The development of bacteremia and meningitis was assessed by taking the appropriate samples 6 hours after the challenge to enumerate the viable bacteria by culture.

Protective antibodies reduce the bacterial numbers, which fully 25 correlates with protection from death within 48 hours (Saukkonen *et al.*, *Microb. Pathog.* 3:261-267 (1987)).

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## Example 4

Immunization of Mice with BacP1.7,16 Protein  
Treated With Detergent and Guanidine Hydrochloride  
(see Table 1)

5

Solubilization of BacP1.7,16-IB Protein

BacP1.7,16-IB protein is wholly soluble in SDS, guanidine hydrochloride and urea, but only partially in sarkosyl or cetylammonium bromide. The protein precipitates if the solubilizing agent is removed. If the 10 agent was guanidine hydrochloride, the precipitated protein is referred to as BacP1.7,16-Gu. In detail BacP1.7,16-Gu is prepared from BacP1.7,16-IB in the following way: 5 mg of BacP1.7,16-IB protein was solubilized with 1 ml of 6 M guanidine hydrochloride. After centrifugation (for 5 min 5,000xg) the clear supernatant was diluted 1:6 in water and dialyzed against water. The 15 precipitate was collected by centrifugation. BacP1.7,16-Gu differs from BacP1.7,16-IB in being fairly soluble both in sarkosyl and cetylammonium bromide (CTB).

Mice were immunized with BacP1.7,16-IB and -Gu preparations, and with preparations dissolved in the above mentioned 20 detergents (1 mg of protein/1 ml of 2% detergent in 10 mM TrisHCl, pH 8.0 containing 5 mM EDTA) and diluted 1:5 in PBS.

The immune sera were analyzed as above. The result of the tests are shown in Table 1. All the sera contained antibodies against BacP1.7,16 protein, but only one of them, HH249 prepared with 25 sarkosyl-solubilized BacP1.7,16-Gu protein, had antibodies against MenB:P1.7,16 protein and was slightly bactericidal and protective.

**Example 5****Immunization of mice with BacP1.7,16-LPS Complexes**

- a) One mg of BacP1.7,16-Gu was solubilized in 1 ml of 3 M guanidine hydrochloride. 250  $\mu$ g of LPS was added and the sample was 5 diluted 1:3 in water and sequentially dialyzed against 0.6 M, 0.3 M and 0.15 M guanidine hydrochloride and 0.15 M NaCl. Before immunization the sample was diluted 1:5 in PBS.
- b) One mg of BacP1.7,16-Gu (as in a)) was solubilized in 1 ml of 1% SDS in 10 mM TrisHCl, pH 8.0 containing 5 mM EDTA. 250  $\mu$ g of 10 LPS was added. The sample was extensively dialyzed against 10 mM Trisbuffer, pH 8.0. Before immunization the sample was diluted 1:5 in PBS.
- c) One mg of BacP1.7,16-Gu (as in a)) was solubilized in 0.1 ml of 1% SDS (as in b)). 250  $\mu$ g LPS and 0.9 ml RIPA buffer (150 mM NaCl, 1% NP-40, 0.5 Doc, 0.1% SDS in 50 mM TrisHCl, pH 8) were added. 15 Before immunization the sample was diluted 1:5 in PBS.

The immune sera were analyzed as using the above methods discussed above. The result of the tests are shown in Table 2. All the sera contained substantial amounts of antibodies against BacP1.7,16 protein. They also had antibodies against MenB:P1.7,16-membranes, were bactericidal and 20 protective, which indicates that protective epitopes were obtained by this refolding method.

**Example 6****Immunization of Mice with BacP1.7,16-Lecithin Complexes**

- 25 a) 1 mg BacP1.7,16-Gu was dissolved in 1 ml of 2% SDS in 100 mM TrisHCl, pH 8, and heated for 5 min. at 100°C. The clear supernatant was diluted 1:5 either with 2% octylglucoside or 2% octyloligoxyethylene (octyl-POE), in 100 mM TrisHCl, pH 9, and incubated overnight at room temperature.
- 30 b) Preparation of a lecithin-detergent film on glass tube. 100 mg of octylglucoside or octyl-POE was dissolved in 2.5 ml of chloroform:methanol (2:1) and 20 mg of soybean lecithin in chloroform was

added. The chloroform was evaporated away under N<sub>2</sub>. Solution (a) was added onto the film. After thorough mixing the suspension was dialyzed against PBS for 2 days with 4 exchanges. Before immunization the sample was diluted 1:5 in PBS.

5           The immune sera were analyzed using the methods disclosed above. The result of the tests are shown in Table 3. All the sera contained antibodies against BacP1.7,16 protein and MenB:P1.7,16-membranes, were bactericidal and protective, which indicates that protective epitopes were obtained by this refolding method.

10

#### Example 7

##### Immunization of Mice with BacP1.7,16 Protein-Lecithin-Sarkosyl Complexes

One mg of BacP1.7,16-IB or BacP1.7,16-Gu was suspended in 1 ml of 2% sarkosyl, and 0.1 ml of soybean lecithin (25 mg/ml of 2% sarkosyl) was added. Before immunization the samples were diluted 1:5 in 0.9% NaCl. In each case the mice received 0.2 ml of the antigen preparation.

15           The immune sera were analyzed using the methods disclosed above. The result of the tests are shown in Table 4. One of the two sera, prepared with BacP1.7,16-IB protein, contained antibodies against BacP1.7,16 protein and MenB:P1.7,16-membranes, was bactericidal and protective. This 20 indicates that protective epitopes were obtained by this refolding method.

#### Example 8

##### Refolding of OmpA by Addition of Lipopolysaccharide

25           Datta *et al.*, *J. Bact.* 131:821-829 (1977) have shown that the bacteriophage K3 receptor loop, in the purified OmpA of *Escherichia coli*, can be refolded with the aid of LPS (lipopolysaccharide) and magnesium. This loop consists the amino acid residues centered in amino acid number 70 and is supposed to be located outside the outer membrane. The refolding 30 was measured by phage-binding inhibition assay; e.g., decrease of the number of plaques titrated on indicator *E. coli* bacteria indicates presence of native epitopes in the refolded BacOmpA.

- It has been shown that BacOmpA<sub>228</sub>OmpA<sub>228</sub>-ss (a tandem duplication of amino acid 8-228 of OmpA with complete signal sequence of *Bacillus amyloliquefaciens* (produced in the strain IH6443) can be refolded similarly as purified OmpA of *E. coli*. The conformation of OmpA
- 5 (BacOmpA-ssΔ) by LPS was studied using BacOmpA<sub>228</sub>OmpA<sub>228</sub>-ss protein as a positive control. The BacOmpA-ssΔ, as shown in Table 5, was able to inhibit the binding of K3 phages to the indicator *E. coli*. The mass needed was, however, more than that of BacOmpA<sub>228</sub>OmpA<sub>228</sub>-ss, as 15 μg of BacOmpA<sub>228</sub>OmpA<sub>228</sub>-ss(+LPS) inhibited 83% of the phage binding, whereas
- 10 75 μg of BacOmpA-ssΔ(+LPS) was needed to inhibit 70% of the phage binding. Hence binding by BacOmpA-ssΔ+LPS is less efficient. It can be said that the phage binding capacity of BacOmpA-ssΔ can be restored with LPS to a limited extent.

15

#### Deposits

Plasmid pKTH290 in *Bacillus subtilis* in the parent strain IH6140 (the recombinant strain denoted as strain IH6627) was deposited on July 5, 1990 with the Deutsche Sammlung von Mikroorganismen (DSM), Mascheroder Weg 1, D-3300 Braunschweig, Federal Republic of Germany, under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under the Treaty. The deposit has been accorded DSM Deposit No. DSM 6089. Samples of *Bacillus subtilis* containing plasmid pKTH290 are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of all nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted.

TABLE 1. Immunization of mice with detergent or guanidine hydrochloride-treated BacP1.7,16 protein.

Scrum	Antigen	Adjuvant	EIA	EIA	Bactericidal titer	Protective titer
5			BacP1.7,16	MenB:p1.7,16 <sup>3</sup>		
HH176	<sup>1</sup> SDS	Al	21100	1100	<1:4	n.d. -
HH177	<sup>1</sup> SDS	FCA	>100000	1390	<1:4	n.d.
HH179	<sup>1</sup> Gu	Al	2880	<1000	<1:4	n.d.
HH180	<sup>1</sup> Gu	FCA	2880	<1000	<1:4	n.d.
HH248	<sup>2</sup> Sarkosyl-IB	FCA	3320	<1000	<1:4	n.d.
HH249	<sup>2</sup> Sarkosyl-Gu	FCA	5880	12200	1:16	1:10
HH279	<sup>2</sup> Sarkosyl-Gu	FCA	22600	1000	1:4	n.d.
HH285	<sup>2</sup> CTB-IB	FCA	19000	1000	1:4	n.d.
HH286	<sup>2</sup> CTB-Gu	FCA	23000	<1000	<1:4	n.d.
HH209	meningococcal	FCA	2400	<100000	1:128	1:100

<sup>1</sup> BacP1.7,16 inclusion bodies (1mg protein) were dissolved in 1 ml of 2% SDS or 5 M guanidine hydrochloride (Gu). After extensive dialysis, the samples were diluted 1:5 in PBS and used for immunization.

<sup>2</sup> One mg off BacP1.7,16 as inclusion bodies (IB) or in the Gu-treated form was suspended in 2% sarkosyl or cetylammonium bromide (CTB) in 10 mM Tris-HCl, pH 8.0.

<sup>3</sup> MenB:p1.7,16-envelopes.

TABLE 2. Immunization of mice with BacP1.7,16-LPS complexes.

Serum	Antigen	Adjuvant	EIA	EIA	Bactericidal	Protective
10		BacP1.7,16	MenB:p1.7,16 <sup>1</sup>	liter	liter	liter
I-II1175	example 5b	none	>100000	13400	1:16	1:10
I-II1178	example 5a	none	>100000	60000	1:128	1:10
I-II1187	example 5a	none	>100000	9750	1:32	1:10
I-II4220	example 5c	none	6220	16170	1:128	1:10

TABLE 3. Immunization of mice with BacP1.7,16-lecithin complexes.

Serum	Antigen	Adjuvant	EIA	EIA	Bactericidal titer	Protective titer
			BacP1.7,16	MenB:p1.7,16 <sup>1</sup>		
HH228	example 6	FCA	3380	14500	1:16	1:10
HH263	example 6	none	2560	10700	1:8	1:10
HH273	example 6	none	1370	8100	1:8	1:10
HH274	example 6	none	2080	11500	1:4	1:10
HH298	example 6	none	14900	100000	1:64	n.d.
HH299	example 6	none	5890	20800	n.d.	n.d.
HH301	example 6	FCA	54300	100000	n.d.	n.d.

<sup>1</sup> MenB:15:P1.7,16-envelopes.

TABLE 4. Immunization of mice with BacP1.7,16-lecithin-sarkosyl complexes.

Serum	Antigen	Adjuvant	EIA	EIA	Bactericidal	Protective
HH290			BacP1.7,16	MenB:p1.7,16 <sup>1</sup>	titer	titer
	BacP1.7,16-IB	FCA	14900	30000	1:128	>1:10
HH291	BacP1.7-16Gu	FCA	<1000	<1000	<1:4	n.d.
	MenB:15:P1.7,16-envelopes.					

MenB:15:P1.7,16-envelopes.

TABLE 5. The inhibition of bacteriophage K3 by BacOmpA<sub>220</sub>OmpA<sub>220</sub>-ss-LPS and BacOmpA-LPS complexes.

Inhibitor	$\mu\text{g}$ of protein and LPS/assay		
	protein	15	40
LPS		45	120
percentage of inhibition of phages binding to the bacteria			
BacOmpA <sub>220</sub> OmpA <sub>220</sub> -ss+LPS		83	93
BacOmpA-ssΔ+LPS		42	47
BacOmpA-ssΔ-LPS (no LPS)		0	nd
LPS, no protein		0.21	0
no LPS, no protein		0	0

### Summary

It may be seen that the invention provides a method for producing cloned and renatured outer membrane (OM) protein from pathogenic gram-negative bacteria. The renatured (OM) proteins produced by the method of the invention have immunologically active epitopes which are capable of eliciting production of antibodies, in mammals and other animals, that are bactericidal and can provide protection against infection by pathogenic gram-negative bacteria. These cloned renatured OM proteins are useful as diagnostic antigens for the identification of infections caused by gram-negative bacteria. They are also useful as vaccines or as components of vaccines.

Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Neisseria meningitidis*
- (B) STRAIN: IH5341

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AACCAAGCTT GATGTCAGCC TGTACGGCGA AATCAAAGCC

40

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Neisseria meningitidis*
- (B) STRAIN: IH5341

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AACCAAGCTT AGAATTGTG GCGCAAAACCG ACGGAGGCC C

41

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1122 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Neisseria meningitidis
- (B) STRAIN: IH5341

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATGTCAGCC	TGTACGGCGA	AATCAAAGCC	GGCGTGGAAAG	GCAGGAACTA	CCAGCTGCAA	60
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ACTAAGGCCA	AAAGCCGCAT	CAGGACGAAA	ATCAGTGATT	TCGGCTCGTT	TATCGGCTTT	180
AAAGGGGAGTG	AGGATTTGGG	CGACGGGCTG	AAGGCTGTTT	GGCAGCTTGA	GCAAGACGTA	240
TCCGTTGCCG	GGCGCGGCCG	GACCCAGTGG	GGCAACAGGG	AATCCTTTAT	CGGCTTGGCA	300
GGCGAATTGCG	GTACGCTGCG	CGCCGGTCGC	GCTGCGAAC	AGTTTGACGA	TGCCAGCCAA	360
GCCATTGATC	CTTGGGACAG	CAATTAATGAT	GTGGCTTCGC	AATTGGGTAT	TTTCAAACGC	420
CACGACGACA	TGCCGGTTTC	CGTACGCTAC	GATTCCCCCG	AATTTTCCGG	TTTCAGCGGC	480
AGCGTTCAAT	TCGTTCCGAT	CCAAAACAGC	AAGTCCGCCT	ATACGCCGGC	TTATTATACT	540
AAAGGATACAA	ACAAATATCT	TACTCTCGTT	CCGGCTGTTG	TGGCGAACGC	CGGATCGGAT	600
GTGTATTATG	CCGGCTCTGA	TTACAAAAAT	GGCGGTTTG	CCGGGAACTA	TGCCTTTAAA	660
TATGCGAGAC	ACGCCAATGT	CGGACGTAAT	GCTTTGAGT	TGTTCTTGAT	CGGCAGCGGG	720
AGTGAATCAAG	CCAAAGGTAC	CGATCCCTTG	AAAAACCATC	AGGTACACCG	TCTGACGGGC	780
GGCTATGAGG	AAGGC GGCTT	GAATCTCGCC	TTGGCGGCTC	AGTTGGATTT	GTCTGAAAAAT	840
GGCGACAAAA	CCAAAACAG	TACGACCGAA	ATTGCCGCCA	CTGCTTCCTA	CCGCTTCGGT	900
AAATGCGAGTTC	CACGCATCAG	CTATGCCCAT	GGTTTCGACT	TTATCGAACG	CGGTAAAAAA	960
GGCGAATATA	CCAGCTACGA	TCAAATCATC	GCCGGCGTTG	ATTATGATTT	TTCCAAACGC	1020
ACTTCCGCCA	TCGTGTCTGG	CGCTTGGCTG	AAACGCAATA	CCGGCATCGG	CAACTACACT	1080
CAAATTAATG	CCGGCGCCGT	CGGTTTGC	GCACAAATTCT	AA		1122

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0 What is claimed is:

1. A method for producing cloned outer membrane protein from pathogenic gram-negative bacteria and renaturing the cloned outer membrane protein thus produced so as to regain immunologically active epitopes which are capable of eliciting production of antibodies, in mammals and other animals, that are bactericidal and capable of providing protection against infection by the original infectious agent, said method comprising: (a) expressing in a gram-positive bacterial host DNA encoding outer membrane protein from gram-negative bacteria known to be pathogenic in humans and animals, (b) renaturing said outer membrane protein from step (a) so as to regain biologically or immunologically active epitopes which are capable of eliciting production of antibodies in animals and humans that are bactericidal and protect said animals and humans from infection by said gram-negative bacteria known to be pathogenic in humans and animals.
2. The method of Claim 1 wherein said bacterial host is any bacterium of the genus *Bacillus*.
3. The method of Claim 1 wherein said host is *Bacillus subtilis*.
4. The method of Claim 1 wherein said DNA encoding said outer membrane protein is selected from the group consisting of DNA sequences encoding outer membrane proteins of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Yersinia sp.* and *Brucella sp.*
5. The method of the Claim 1 wherein said DNA sequence encoding said outer membrane protein is a DNA sequence encoding class 1 outer membrane protein of *Neisseria meningitidis*.
6. The method of Claim 1 wherein said outer membrane protein from step (a) is renatured with an agent or combination of agents selected from the group consisting of SDS, guanidine hydrochloride, cetylammonium bromide, phospholipids, lecithin, sarkosyl and urea.
7. Outer membrane protein of pathogenic gram-negative bacteria prepared by any of the methods of Claims 1-6.

8. Outer membrane protein of pathogenic gram-negative bacteria, wherein the outer membrane protein is class 1 outer membrane protein of *Neisseria meningitidis*, prepared by any of the methods of Claims 1-6.

5 9. Outer membrane protein of pathogenic gram-negative bacteria, wherein the outer membrane protein is class 1 outer membrane protein of *Neisseria meningitidis*, and further wherein said class 1 outer membrane protein is from a strain of *Neisseria meningitidis* which belongs to serogroup B, prepared by any of the methods of Claims 1-6.

10 10. A composition comprising cloned and renatured outer membrane protein produced by any of the methods of Claims 1-6.

11. A composition comprising cloned and renatured outer membrane protein produced by any of the methods of Claims 1-6, wherein said composition is in a pharmaceutically acceptable dosage form.

15 12. A vaccine comprising cloned and renatured outer membrane protein produced by any of the methods of Claims 1-6.

13. A vaccine according to Claim 12 wherein said vaccine also contains immunoadjuvants and pharmacologically-acceptable preservatives.

20 14. Use of outer membrane proteins of any of Claims 7-10 as antigenic diagnostic reagents for detecting antibodies against pathogenic gram-negative bacteria with immunological methods.

15. Use of outer membrane proteins of any of Claims 7-10 as a vaccine or component thereof.

25 16. A method for producing cloned outer membrane protein from pathogenic gram-negative bacteria and renaturing the cloned outer membrane protein thus produced so as to regain immunologically active epitopes which are capable of eliciting production of antibodies, in mammals and other animals, that are bactericidal and capable of providing protection against infection by the original infectious agent, said method comprising: (a) expressing in a gram-positive bacterial host DNA encoding outer membrane protein from class 1 outer membrane protein of *Neisseria meningitidis* (b) renaturing said outer membrane protein from step (a) so as to regain

biologically or immunologically active epitopes which are capable of eliciting production of antibodies in animals and humans that are bactericidal and protect said animals and humans from infection by *Neisseria meningitidis*.

17. The method of Claim 16 wherein said bacterial host is any  
5 bacterium of the genus *Bacillus*.

18. The method of Claim 16 wherein said host is *Bacillus subtilis*.

19. The method of Claim 16 wherein said outer membrane protein from step (a) is renatured with an agent or combination of agents  
10 selected from the group consisting of SDS, guanidine hydrochloride, cetylammonium bromide, phospholipids, lecithin, sarkosyl and urea.

20. Class 1 outer membrane protein of *Neisseria meningitidis*, prepared by any of the methods of Claims 16-19.

21. Class 1 outer membrane protein of *Neisseria meningitidis*,  
15 wherein said class 1 outer membrane protein is from a strain of *Neisseria meningitidis* which belongs to serogroup B, prepared by any of the methods of Claims 16-19.

22. A composition comprising cloned and renatured outer membrane of *Neisseria meningitidis* produced by any of the methods of Claims  
20 16-19.

23. A composition comprising cloned and renatured outer membrane protein of *Neisseria meningitidis* produced by any of the methods of Claims 16-19, wherein said composition is in a pharmaceutically acceptable dosage form.

25 24. A vaccine comprising Class 1 outer membrane protein of *Neisseria meningitidis*, prepared by any of the methods of Claims 16-19.

25. A vaccine according to Claim 24 wherein said vaccine also contains immunoadjuvants and pharmacologically-acceptable preservatives.

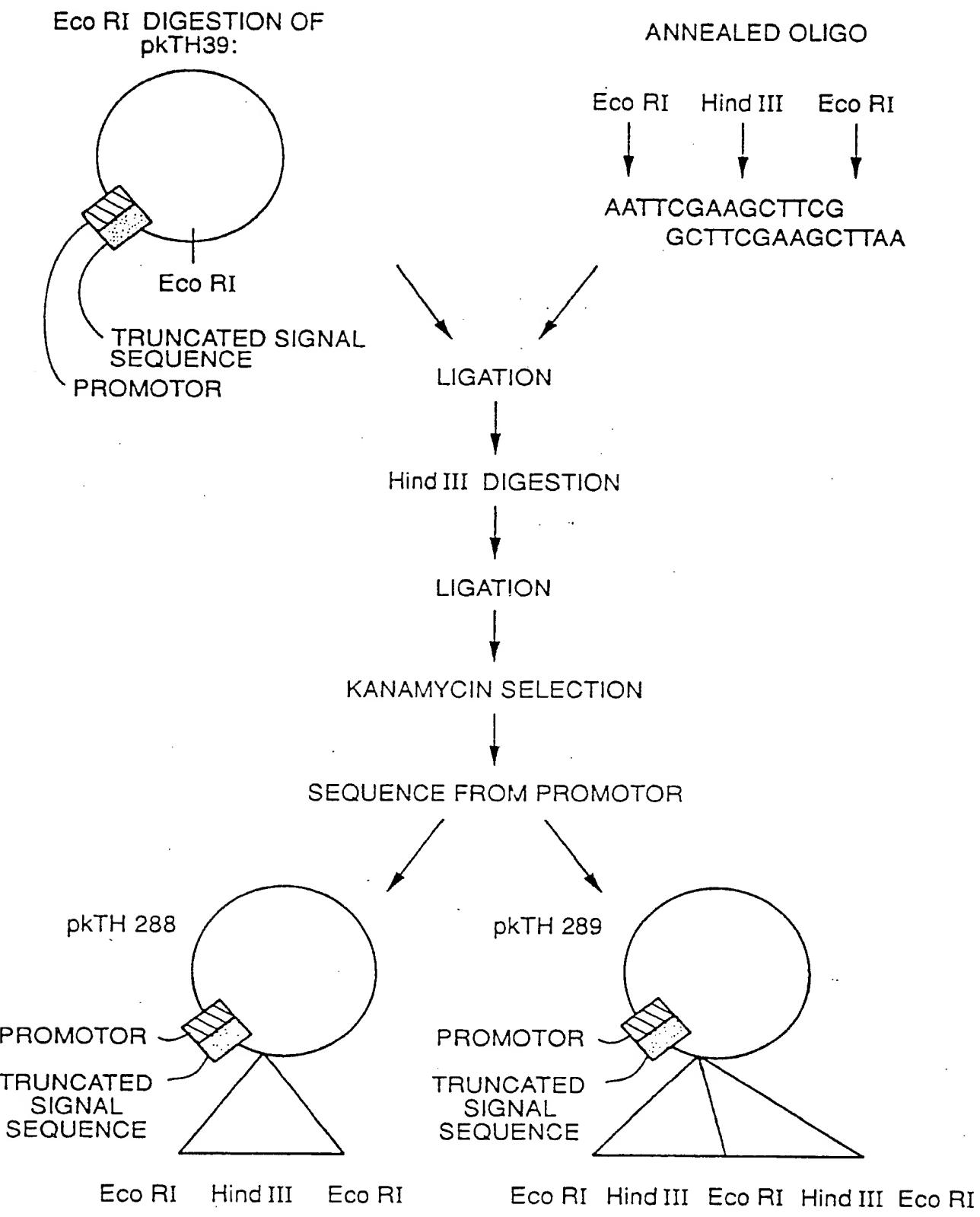
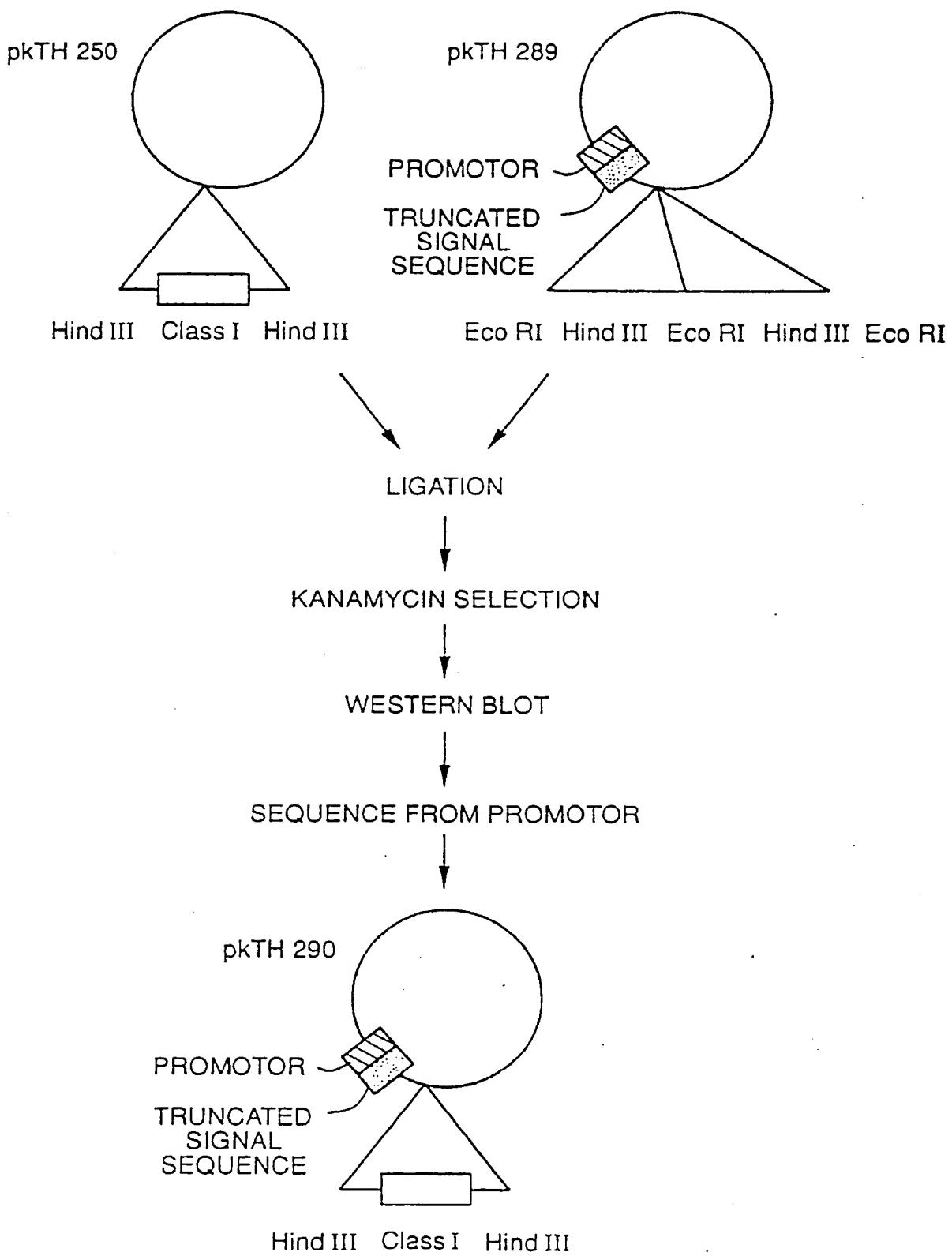


FIG.\_1

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## Hind III DIGESTION OF:

***FIG.\_2***

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

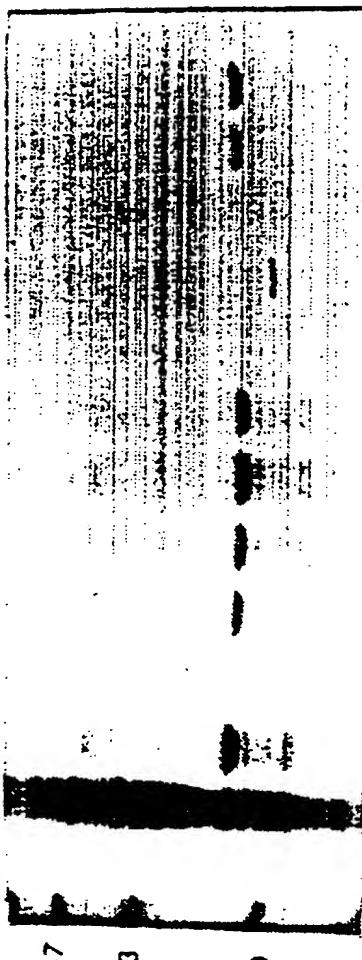
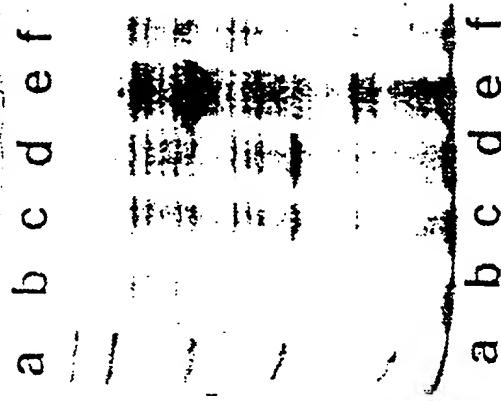


FIG.-3



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1 2 3 4 5 6 7 8 9 10 - 11 - 12 - 13 - 14

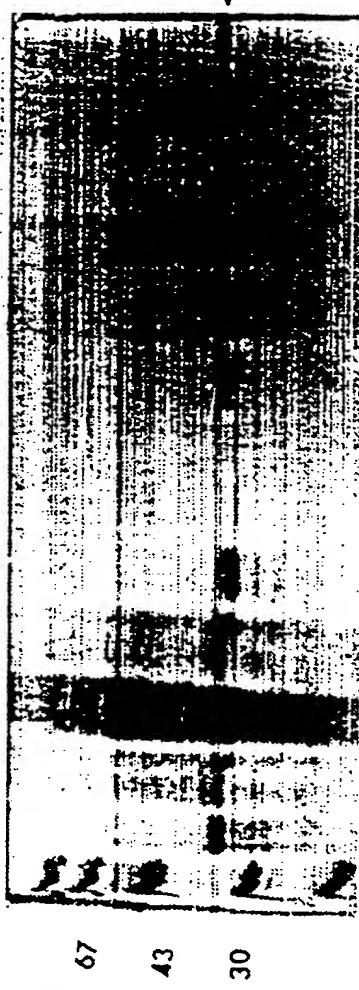


FIG.-4

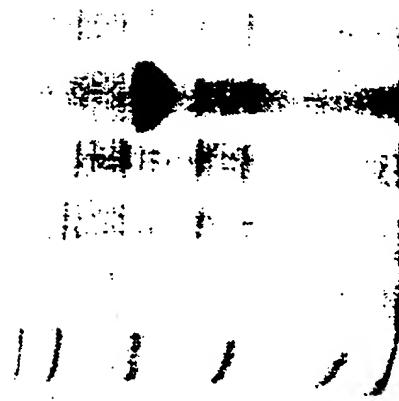


FIG.-6

## INTERNATIONAL SEARCH REPORT

International Application No PCT/FI 91/00212

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC5: C 07 K 15/04, A 61 K 39/095, 39/10, 39/102, 39/02  
 C 12 N 15/31, C 12 N 15/75

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
IPC5	C 07 K; A 61 K; C 12 N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in Fields Searched<sup>8</sup>

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07349245, Puohiniemi R et al: "A strong antibody response to the periplasmic C-terminal domain of the OmpA protein of Escherichia coli is produced by immunization with purified OmpA or with whole E. coli or Salmonella typhimurium bac- teria", & Infect Immun Jun 1990, 58 (6) p1691-6	1-3,6-7, 10-15
Y	--	1-25
X	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 05979348, Kallio P et al: "Synthesis of OmpA protein of Escherichia coli K 12 in Bacillus subtilis", & J Gen Microbiol Mar 1986, 132 (Pt 3) p677-87	1-3,6-7, 10-15
Y	--	1-25

<sup>\*</sup> Special categories of cited documents:<sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

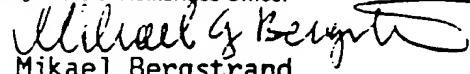
1st October 1991

Date of Mailing of this International Search Report

1991 -10- 03

International Searching Authority

Signature of Authorized Officer


 Mikael Bergstrand

SWEDISH PATENT OFFICE

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07371344, McGuinness B et al: "Deduced amino acid sequences of class i protein (PorA) from three strains of <i>Neisseria meningitidis</i> . Synthetic peptides define the epitopes responsible for serosubtype specificity", & J Exp Med Jun 1 1990 171 (6) p1871-82 --	1-25
Y	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 06768281, Tarkka E et al: "Cloning of an outer membrane protein of <i>Neisseria meningitidis</i> in <i>Escherichia coli</i> ", & Microb Pathog Dec 1987, 3 (6) p445-53 --	1-4,7, 10-15
Y	Dialog Information Services, File 154, Medline 85-91 dialog accession no. 06452432, Carbonetti NH et al: "Molecular cloning and characterization of the structural gene for protein I, the major outer membrane protein of <i>Neisseria gonorrhoeae</i> ", & Proc Natl Acad Sci USA Dec 1987, 84 (24) p9084-8 --	1-4,7, 10-15
Y	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 06777316, Munson R Jr et al: "Molecular cloning, expression, and primary sequence of outer membrane protein P2 of <i>Haemophilus influenzae</i> type b", & Infect Immun Jan 1989, 57 (1) p88-94 --	1-4,7, 10-15
Y	WO, A1, 8807374 (THE TEXAS A & M UNIVERSITY SYSTEM) 6 October 1988, see the whole document --	1-4,2; 10-15
Y	WO, A1, 8804932 (PRAXIS BIOLOGICS, INC.) 14 July 1988, see the whole document -----	1-4,7, 10-15

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.PCT/FI 91/00212

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the Swedish Patent Office EDP file on **91-08-30**.  
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 8807374	88-10-06	AU-D- 1573288 EP-A- 0357642 GB-A- 2227936 JP-T- 2503196	88-11-02 90-03-14 90-08-15 90-10-04
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WO-A1- 8804932	88-07-14	AU-D- 1154188 EP-A- 0294469	88-07-27 88-12-14

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